APPENDIX:

The Appendix includes the following items:

☒ - a Declaration

☒ - Exhibits 1-6

PATENT

0546-1082

IN THE U.S. PATENT AND TRADEMARK OFFICE

In re application of

Steve HEALD et al.	Conf. 4057
Application No. 10/550,945	Group 1651
Filed November 30, 2006	Examiner Irene Marx

PREPARATION OF VANILLIN FROM MICROBIAL TRANSFORMATION MEDIA BY EXTRACTION BY MEANS SUPERCRITICAL FLUIDS OR GASES

DECLARATION

Assistant Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

Sir:

1. I, **Mylène DARRICAU**, am a French citizen. I am employed by the French firm SAFISIS (a subsidiary of LESAFFRE), in the capacity of R&D Project Manager, and have held that position since January 5th, 2009. My background is a Master of Engineering in Biotechnology with 7 years of experience in Fermentation and Microbiology; I have a diploma from Engineering School of INSA in Toulouse (France), and I am in charge of Research and Development projects for SAFISIS in Biomolecules.

   I, **Evelyne FONCHY-PENOT**, hereby declare that I am a French citizen. I am employed by the French firm LESAFFRE INTERNATIONAL (a subsidiary of LESAFFRE), in the capacity of R&D Project Manager, and have held that position since 2000. LESAFFRE INTERNATIONAL is a service company which supports the different subsidiaries of LESAFFRE particularly in Research projects.

My background is PhD in biotechnology with more than 10 years experience in fermentation and Microbiology; I have a diploma from Engineer School from ESIL in Marseille (France), and I am in charge of Research and Development projects for LESAFFRE INTERNATIONAL in Biomolecules and Bioprocess.

2. We are familiar with the above-identified U.S. patent application, its prosecution before the United States Patent and Trademark Office, and the applied references of Rabenhorst et al. (U.S. Patent No. 6,133,003), Muheim et al. (U.S. Patent No. 6,235,507) and Makin (U.S. Patent No. 4,474,994).

3. In order to demonstrate the patentability of the present invention, we are submitting the following observations.

We confirm hereby that we have conducted the following experiments.

We state that, since my company got reports from independent laboratories that compare DSM 9991 and DSM 9992 of H&R patent and conclude that these two strains cannot be distinguished from one another; experiments were conducted on the sole DSM 9992 strain, exactly as disclosed in the H&R patent Examples 1 and 3.

We, Mrs DARRICAU and Mrs FONCHY-PENOT, who are researchers at SAF-ISIS and LESAFFRE INTERNATIONAL respectively, have realized the works detailed in the enclosed report (Exhibit 1)

As a summary the conclusions are that:

Strains DSM 9992 from US Patent No. 6,133,003 and Zyl 926 from the SAF-ISIS present application do not react at all the same way when applying, the same process as disclosed in examples 1 and 3 of US Patent No. 6,133,003.

Moreover, the HPLC analysis controls show that the ferulic acid was accumulated in the wort so that no bioconversion occurred with any of these two strains.

This means that the process in the US Patent No. 6,133,003is inoperatively disclosed in order to get the alleged results regarding vanillin production.

As a consequence we state that the prior art was duly worked out with the intention to reduce to practice the working examples of US Patent No. 6,133,003, with the above mentioned absence of results.

We state that we made all reasonable due efforts, that a person of ordinary skill in the art should make, by taking all the information given in the specification such as for instance pH and oxygen maintenance, and also by using our general knowledge for example, to control the foaming that occurred during the fermentation by adding an antifoam.

Accordingly, we consider that, contrary to the DSM 9991 and 9992 strains of US Patent No. 6,133,003, the Zyl 926 microorganism, of the present application of SAF-ISIS, deposited within CABI as IMI 390106 for the purposes of patent procedures under the Budapest Treaty, provides unexpected results.

The latter are demonstrated notably by:
-a consumption of ferulic acid with concomitant production of above 11 g/L of vanillin
-a vanillin product obtained there from absent of odoriferous by-products and with none of guaïacol, vinyl guaïacol, eugenol and isoeugenol being present at more than 100ppm.

4. We declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date June 29, 2010

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Exhibit 1

Report on the "Characterisation of Strains belonging to the Genus *Amycolatopsis* by 16S rRNA Gene Sequencing"

Professor M. Goodfellow

Background

Initially, the client wished to have the taxonomic relationships between the three *Amycolatopsis* strains determined by 16S rRNA gene sequencing. This study was designed to establish the inferred phylogenetic relationships between the cultures and their position (s) in the *Amycolatopsis* 16S rRNA gene tree.

Receipt of Strains

The package containing the strains arrived intact on May 20th (See photographs attached).

Materials and Methods

Cultivation of strains. The strains were grown in shake flasks (150 rpm) in ISP 2 broth for 48 hours at 37°C. Following incubation, biomass was harvested by centrifugation.

16S rRNA gene sequencing analyses. Chromosomal DNA was isolated and 16S rRNA PCR was done using well established procedure (Lane, 1991). PCR product was purified and direct sequencing of 16S rRNA genes of the three *Amycolatopsis* strains was carried out using BigDyeTM version 3.1 Terminator Cycle Sequencing Kit (PE Applied Biosystems, MA). Samples were completed using 10 ng of DNA for every 100 bp of template DNA and 3.2 pmol of primer. Post-sequencing reactions were purified away from unincorporated dye terminators using Performa DTR spin-columns (EdgeBiosystems, MD). Sequences were determined using an ABI 3730 Sequencer. The resultant almost complete 16S rRNA gene sequences (1360 nucleotides) were aligned manually against corresponding sequences of the type strains of *Amycolatopsis* species, retrieved from GenBank, using PHYDIT software (Chun, 1995). A phylogenetic tree was inferred by using neighbour-joining (Saitou & Nei, 1987) tree-making algorithm from the PHYDIT program and an evolutionary distance matrix generated by using the distance model of Jukes & Cantor (1969). The robustness of the phyletic lines recovered in the *Amycolatopsis* tree were determined in a bootstrap analysis (Felsenstein, 1985) of the neighbour-joining dataset by using the SEQBOOT and CONSENSE options from the PHYLIP suite of programs (Felsenstein, 1993).

Results

It is apparent from Figures 1 to 3 that the 16S rRNA gene sequences obtained for the three *Amycolatopsis* strains are of high quality as there are no ambiguities in the conserved regions of the traces. It is clear from the 16S rRNA *Amycolatopsis* tree that the three strains belong to the *Amycolatopsis methanolica* subclade, the taxonomic integrity of the latter is underpinned by a 100% bootstrap value (Fig. 4). It is also apparent from the tree and from the associated nucleotide similarity and difference

matrix (Table 1) that the three strains share identical 16S rRNA gene sequences with one another.

Conclusions

- The three *Amycolatopsis* strains belong to the *Amycolatopsis methanolica* subclade in the 16S rRNA *Amycolatopsis* gene tree.

- The three *Amycolatopsis* strains shared identical 16S rRNA gene sequences with one another.

References

Chun, J. (1995). Computer-assisted classification and identification of actinomycetes. Ph.D. Thesis, University of Newcastle, Newcastle upon Tyne, UK.

Felsenstein, J. (1985). Confidence limits on phylogeny: An appropriate use of the bootstrap. *Evolution* **39**, 7783-7791.

Felsenstein, J. (1993). PHYLIP- Phylogenetic Inference Package version 3.5.1. Department of Genetics, University of Washington, Seattle, USA.

Jukes, T.H. & Cantor, C.R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, volume **3**, pp. 21-132. Edited by H. N. Munro, Academic Press, New York.

Lane, D.J. (1991). 16S/23S rRNA Sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115-148. Edited by E. Stackebrandt & M. Goodfellow. John Wiley & Sons, Chichester, New York.

Saitou, N. & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406-425.

Attachments with the Report

Legends for Figures.

Figs. 1a-3b. Chromatograms of Samples A, B and C respectively (a- sequencing with forward primer; b- sequencing with reverse primer).

Fig. 4. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between the three *Amycolatopsis* strains and between them and the type strains of *Amycolatopsis* species. Numbers at the nodes indicate the level of bootstrap support (%) based on neighbour-joining analysis of 1000 resampled datasets; only values above 50% are given. The scale bar indicates 0.02 substitutions per nucleotide position.

Photographs.

Photographs 1-5. Receipt of strains.

Tables.

Table 1. Nucleotide similarity and difference matrix with the three strains which share identical 16S rRNA gene sequences with one another.

M. Goodfellow

Professor Michael Goodfellow

Exhibit 2

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Report No: MB/REP/MMR/115732/00002-00004/2
Report Date: 30/06/09

CONFIDENTIAL
CERTIFICATE OF ANALYSIS
Microbiology Department
MicroID

Notes

The results provided herein relate only to the items tested.

RiboPrint®, RiboGroup® and RiboPrinter™ are trademarks of Qualicon™ L.L.C., a subsidiary of E.I. DuPont de Nemours and Company, Wilmington, Delaware, USA.

Prepared/Checked By:

Authorised By:

Authorised	C. Mitchell Research Technical Officer	
Signatories:	S. Jordan Molecular Methods Manager	
	C. Baylis Methods Research Manager	✓

Any opinions and interpretations contained in this report are outside the scope of UKAS accreditation.

Summary

Three bacterial isolates were sent to Campden BRI from SAFISIS for strain comparison using the Qualicon™ RiboPrinter® at the request of the client. The parcel was received on site unopened as shown in the images taken of the package in Appendix 1.

The isolates were stored and grown prior to analysis as detailed in the NDA supplied by the client. Standard techniques and reagents were used for the ribotyping. The enzyme PvuII was used for the analysis of the isolates as it is has been shown to differentiate between DuPont database entries for *Streptomyces* sp. The results of the work are noted in Tables below, with a pattern comparison shown in Figure 1. A summary of the RiboPrinter methodology is outlined in Appendix 2.

CCFRA Code:	MMR/115732/00002	**Date of Receipt:**	20/05/09
Customer Code	Sample A	**Client presumptive identification**	*Amycolatopsis* sp
Sample Description:	Frozen culture		
Comments:	Stored at ambient temperature prior to analysis		
Analysis Type	**Test Details**	**Identification**	**Comments**
RiboPrinter®	*Pvu*II	None	Isolate fell outside of 0.85 similarity needed for automatic identification against the DuPont identification database. The isolate was placed in RiboGroup 114-3210-S-1.
RiboPrinter®	Strain comparison		Refer to Figure 1 for details

CCFRA Code:	MMR/115732/00003	**Date of Receipt:**	20/05/09
Customer Code	Sample B	**Client presumptive identification**	*Amycolatopsis* sp
Sample Description:	Freeze Dried Ampoule		
Comments:	Stored at ambient temperature prior to analysis		
Analysis Type	**Test Details**	**Identification**	**Comments**
RiboPrinter®	*Pvu*II	None	Isolate fell outside of 0.85 similarity needed for automatic identification against the DuPont identification database. The isolate was placed in RiboGroup 114-3210-S-1.
RiboPrinter®	Strain comparison		Refer to Figure 1 for details

CCFRA Code:	MMR/115732/00004	**Date of Receipt:**	20/05/09
Customer Code	Sample C	**Client presumptive identification**	*Amycolatopsis* sp
Sample Description:	Freeze Dried Ampoule		
Comments:	Stored at ambient temperature prior to analysis		

Analysis Type	**Test Details**	**Identification**	**Comments**
RiboPrinter®	*Pvu*II	None	Isolate fell outside of 0.85 similarity needed for automatic identification against the DuPont identification database. The isolate was placed in RiboGroup 114-3210-S-1.
RiboPrinter®	Strain comparison		Refer to Figure 1 for details

Strain comparison

A comparison of the patterns generated for the three isolates is shown below in Figure 1a and b.

Figure 1a: Strain comparison of isolates MB/115732/2-4 using MB/115732/2 as the reference isolate

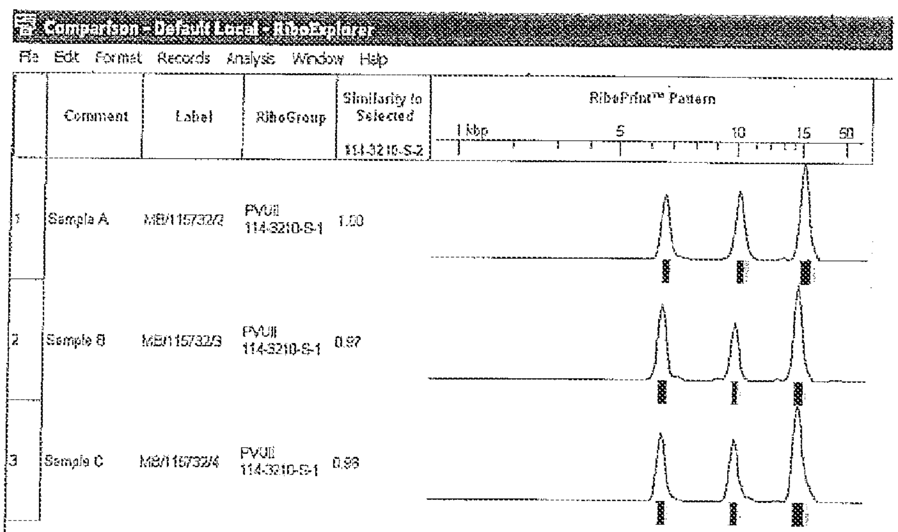
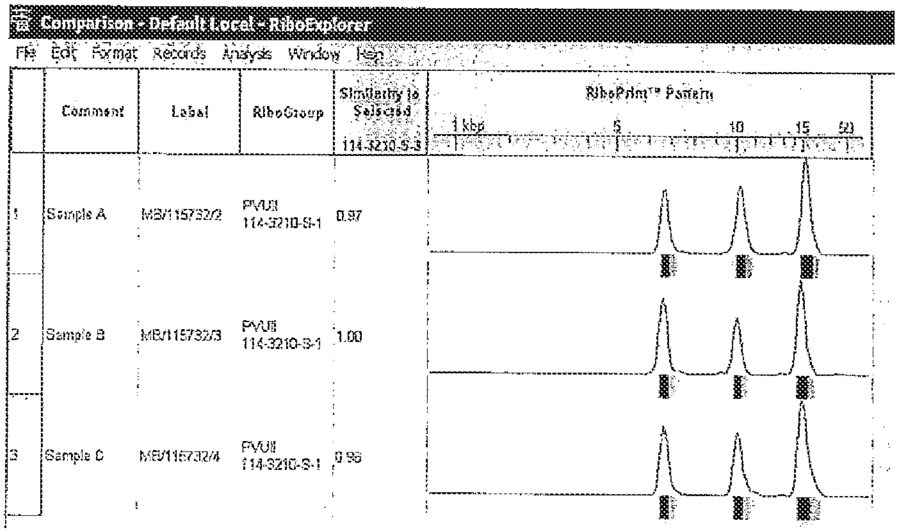


Figure 1b: Strain comparison of isolates MB/115732/2-4 using MB/115732/3 as the reference isolate

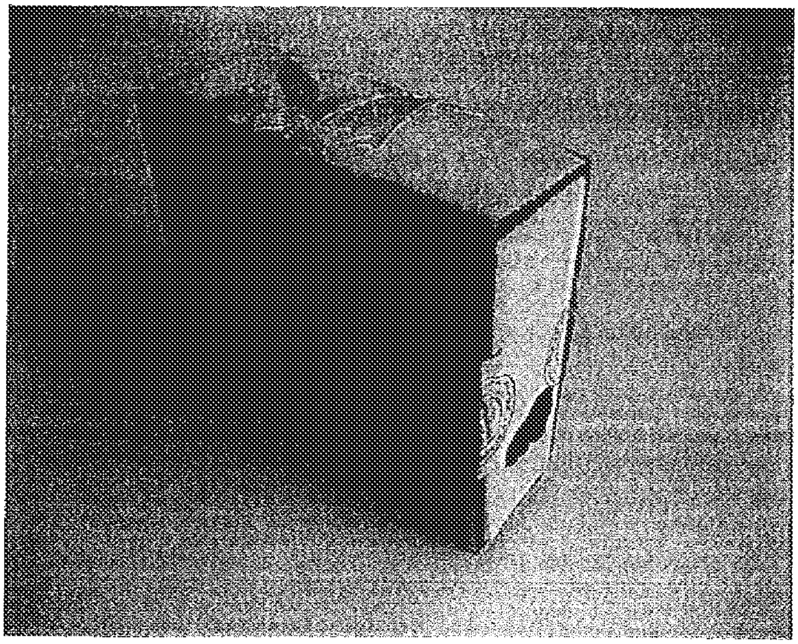


All three isolates were placed in the same Ribogroup 114-3210-S-1. These results suggest that the isolates are indistinguishable from each other using PvuII.

These data readily demonstrate the ability of the RiboPrinter system to characterise and discriminate bacterial strains by analysis of RiboPrint patterns. This information will be available for reference on the archived database and as such can be used to authenticate, or compare to, these cultures time and time again.

These isolates will be kept for a standard period of 4 weeks after ribotyping after which they will be destroyed as outlined in the NDA supplied by the client.

Appendix 1: Image of package received for analysis at Campden BRI



Appendix2: Details of RiboPrinter Methodology

The RiboPrinter system is an automated ribotyping process as follows:

Bacterial cells are broken open to release the genomic DNA into solution. This DNA is digested ('cut up') using a restriction enzyme to generate fragments of different lengths, which are then separated out on the basis of size. Ribosomal DNA is detected using probes which give off light that is captured by a camera. The resulting image is converted into a RiboPrint pattern that can be characterised (into RiboGroups) and where possible identified by proprietary software. RiboGroups are assigned to patterns in the following format - RiboGroup library - Machine number - batch number and sample position where the pattern occurred for the first time for example RIBO1 114-1002-S-1

As each sample is analysed by the software, it makes a decision about how closely the new sample pattern matches the existing patterns in the database. If the software finds a match, that is if it is unable to distinguish the new pattern from an existing pattern (i.e. >92% similarity between patterns), it assigns the new sample to that RiboGroup. All samples in a RiboGroup have the same RiboGroup code. Thus if the isolate has been previously ribotyped on this instrument, the instrument database will recognise the reproduced RiboPrint and allocate the RiboGroup code assigned in the first instance.

When an isolate is analysed on the machine for the first time, i.e. it does not match with any existing patterns, it will receive a new code i.e. the batch and sample number specific to that new batch run.

For automatic (DuPont) identification, the new sample information is compared to existing patterns within a supplied (DUP) database. If the software finds a match of >85% a DuPont identification will be reported.

If no automatic identification is given, the user can instruct the software to show the percentage similarities between existing patterns in the instrument sample database (Campden BRI database) and the new sample pattern. When >85% similarity is shown identification can be offered.

Exhibit 3

PHYDIT - Similarity analysis
1360 nucleotides analysed
Lower-left triangle contains [NT] Similarity.
Uppper-right triangle contains [NT] Different/Total nucleotides.

The following table contains tab-delimited numbers. Copy and paste to MS Excel or Word.

	Amycolatopsis Sample A
Amycolatopsis Sample A	---
Amycolatopsis Sample B	100
Amycolatopsis Sample C	100
Amycolatopsis methanolica DSM 44096T (AJ249135)	98.88
Amycolatopsis thermoflava DSM 44574T (AF052390)	98.88
Amycolatopsis jejuensis NRRL B-24427T (DQ000200)	94.25
Amycolatopsis sulphurea DSM 46092T (AJ293756)	95.37
Amycolatopsis alba DSM 44262T (AF051340)	94.76
Amycolatopsis coloradensis DSM 44225T (AJ293753)	94.47
Amycolatopsis regifaucium DSM 45072T (AY129760)	94.84
Amycolatopsis azurea DSM 43854T (AJ400709)	94.69
Amycolatopsis orientalis DSM 40040T (AJ400711)	94.69
Amycolatopsis keratiniphila subsp. keratiniphila DSM 4440T (AJ278496)	94.69
Amycolatopsis keratiniphila subsp. nogabecina DSM 445T (AJ508238)	94.69
Amycolatopsis japonica DSM 44213T (AJ508236)	94.54
Amycolatopsis decaplanina DSM 44594T (AJ508237)	94.77
Amycolatopsis minnesotensis NRRL B-24435 (DQ076482)	94.84
Amycolatopsis nigrescens DSM 44992T (DQ486888)	95.44
Amycolatopsis lexingtonensis DSM 44653T (AY183358)	94.61
Amycolatopsis pretoriensis DSM 44654T (AY183356)	94.54
Amycolatopsis rifamycinica DSM 46095T (AY083603)	94.69
Amycolatopsis kentuckyensis DSM 44652T (AY183357)	94.54
Amycolatopsis balhimycina DSM 44591T (AJ508239)	94.47
Amycolatopsis vancoresmycina DSM 44592T (AJ508240)	94.84
Amycolatopsis plumensis DSM 44776T (AY262825)	94.61
Amycolatopsis tolypomycina DSM 44544T (AJ293757)	94.61
Amycolatopsis mediterranei DSM 43304T (AJ293754)	94.84
Amycolatopsis australiensis DSM 44671T (AY129753)	95.14
Amycolatopsis saalfeldensis DSM 44993T (DQ792500)	95.07
Amycolatopsis echigonensis JCM 21831T (AB248535)	94.84
Amycolatopsis niigatensis JCM 21832T (AB248537)	95.06
Amycolatopsis benzoatilytica DSM 43387T (AY957506)	93.39
Amycolatopsis albidoflavus DSM 44639T (AJ252832)	94.9
Amycolatopsis halotolerans NRRL B-24428T (DQ000196)	95.21
Amycolatopsis rubida DSM44637T (AF222022)	94.51
Amycolatopsis sacchari DSM 44468T (AF223354)	95.29
Amycolatopsis taiwanensis NBRC 102103T (DQ160215)	94.99
Amycolatopsis marina NBRC 104263T (EU329845)	95.96
Amycolatopsis palatopharyngis JCM 12460T (AF479268)	96.19
Amycolatopsis fastidiosa DSM 43855T (AB184566)	92.38

Amycolatopsis Sample B	Amycolatopsis Sample C
0/1338	0/1338
---	0/1338
100	---
98.88	98.88
98.88	98.88
94.25	94.25
95.37	95.37
94.76	94.76
94.47	94.47
94.84	94.84
94.69	94.69
94.69	94.69
94.69	94.69
94.69	94.69
94.54	94.54
94.77	94.77
94.84	94.84
95.44	95.44
94.61	94.61
94.54	94.54
94.69	94.69
94.54	94.54
94.47	94.47
94.84	94.84
94.61	94.61
94.61	94.61
94.84	94.84
95.14	95.14
95.07	95.07
94.84	94.84
95.06	95.06
93.39	93.39
94.9	94.9
95.21	95.21
94.51	94.51
95.29	95.29
94.99	94.99
95.96	95.96
96.19	96.19
92.38	92.38

Amycolatopsis methanolica DSM 44096T (AJ249135)
15/1338
15/1338
15/1338

99.85
94.32
94.99
94.69
94.47
94.39
94.62
94.54
94.77
94.61
94.47
94.92
94.54
95.74
94.61
94.54
94.62
94.47
94.39
94.47
94.17
94.24
94.76
94.99
95.22
94.54
94.76
92.93
94.75
94.91
94.81
95.74
95.44
96.34
96.49
92.38

Amycolatopsis thermoflava DSM 44574T (AF052390)
15/1337
15/1337
15/1337
2/1337

94.24
94.99
94.61
94.39
94.31
94.54
94.47
94.69
94.54
94.39
94.84
94.47
95.66
94.54
94.47
94.54
94.39
94.32
94.39
94.09
94.16
94.68
94.91
95.14
94.46
94.68
92.85
94.67
94.83
94.73
95.66
95.36
96.26
96.41
92.3

Amycolatopsis jejuensis NRRL B-24427T (DQ000200)
77/1338
77/1338
77/1338
76/1338
77/1337

97.83
96.86
96.64
97.08
97.01
96.79
96.64
96.86
97.01
96.79
96.71
96.71
96.56
96.64
96.64
96.41
96.19
96.64
96.34
96.78
96.48
97.01
97.16
97.23
97.16
95.29
97
96.86
96.77
96.64
93.88
95.44
95.52
90.44

Amycolatopsis sulphurea DSM 46092T (AJ293756)	Amycolatopsis alba DSM 44262T (AF051340)
62/1338	70/1337
62/1338	70/1337
62/1338	70/1337
67/1338	71/1337
67/1337	72/1336
29/1339	42/1337
---	45/1337
96.63	---
96.49	99.25
97.38	98.28
97.01	98.65
96.94	98.58
96.79	98.43
97.01	98.5
96.94	98.28
97.31	98.35
97.01	97.38
97.09	97.53
96.63	97.83
96.56	97.91
96.56	97.98
96.34	97.75
96.26	97.68
96.71	97.68
96.11	97.16
96.41	97.6
96.41	97.9
97.09	97.46
97.16	97.83
97.31	97.38
97.68	97.53
95.67	95.82
97.68	97.6
97.68	97.53
96.92	97.22
97.23	96.19
93.58	94.09
95.52	95.14
95.67	95.44
91.04	91.1

Amycolatopsis coloradensis DSM 44225T (AJ293753)
74/1338
74/1338
74/1338
74/1338
75/1337
45/1338
47/1338
10/1337

98.5
98.58
98.8
98.65
98.65
98.65
98.58
97.61
97.38
97.83
97.91
97.83
97.61
97.68
97.46
97.08
97.53
97.68
97.46
97.91
97.08
97.23
95.59
97.45
97.23
97.14
95.89
93.87
95.07
95.37
90.81

Amycolatopsis regifaucium DSM 45072T (AY129760)
69/1337
69/1337
69/1337
75/1337
76/1336
39/1337
35/1337
23/1336
20/1337

98.88
99.1
98.88
99.25
99.48
99.1
97.46
97.01
97.46
97.38
97.38
97.16
97.01
97.46
97.01
97.38
97.3
97.83
97.23
97.83
98.13
96.43
98.05
98.13
97.29
95.96
93.34
95.29
95.29
91.17

Amycolatopsis azurea DSM 43854T (AJ400709)	Amycolatopsis orientalis DSM 40040T (AJ400711)
71/1338	71/1338
71/1338	71/1338
71/1338	71/1338
72/1338	73/1338
73/1337	74/1337
40/1338	43/1338
40/1338	41/1338
18/1337	19/1337
19/1338	16/1338
15/1337	12/1337
---	6/1338
99.55	---
99.18	99.33
99.48	99.63
99.1	99.18
99.18	99.33
97.16	97.31
97.31	97.31
98.13	97.83
98.06	97.76
97.91	97.61
97.68	97.38
97.68	97.38
97.53	97.38
96.93	96.86
97.46	97.38
97.75	97.46
97.76	97.76
97.76	97.46
97.61	97.38
97.9	97.68
96.43	96.35
97.9	97.83
97.75	97.68
97.67	97.44
96.26	96.26
93.87	93.65
95.74	95.59
96.04	95.81
91.63	91.63

Amycolatopsis keratiniphila subsp. keratiniphila DSM 4440T (AJ278496)
71/1338
71/1338
71/1338
70/1338
71/1337
45/1338
43/1338
21/1337
18/1338
15/1337
11/1338
9/1338

99.7
99.25
99.33
97.31
97.53
97.83
97.76
97.46
97.23
97.23
97.46
96.86
97.31
97.38
97.61
97.38
97.76
97.9
96.05
97.98
97.75
97.89
96.49
93.87
95.89
96.11
91.55

Amycolatopsis keratiniphila subsp. nogabecina DSM 445T (AJ508238)
71/1337
71/1337
71/1337
72/1337
73/1336
42/1337
40/1337
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91.77

Amycolatopsis japonica DSM 44213T (AJ508236)	Amycolatopsis decaplanina DSM 44594T (AJ508237)
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73/1338	70/1338
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97.76	97.31
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97.38	97.08
97.23	96.94
97.31	97.09
96.93	96.48
97.46	96.93
97.46	97.31
97.68	97.98
97.38	97.09
97.53	97.68
97.75	98.13
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97.75	97.98
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97.37	97.67
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95.52	95.89
95.59	95.81
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Amycolatopsis minnesotensis NRRL B-24435 (DQ076482)
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Amycolatopsis nigrescens DSM 44992T (DQ486888)
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Amycolatopsis lexingtonensis DSM 44653T (AY183358)
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Amycolatopsis pretoriensis DSM 44654T (AY183356)
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Amycolatopsis rifamycinica DSM 46095T (AY083603)
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Amycolatopsis kentuckyensis DSM 44652T (AY183357)
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Amycolatopsis balhimycina DSM 44591T (AJ508239)
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Amycolatopsis vancoresmycina DSM 44592T (AJ508240)
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Amycolatopsis plumensis DSM 44776T (AY262825)
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Amycolatopsis tolypomycina DSM 44544T (AJ293757)
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Amycolatopsis mediterranei DSM 43304T (AJ293754)
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Amycolatopsis australiensis DSM 44671T (AY129753)
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Amycolatopsis saalfeldensis DSM 44993T (DQ792500)
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Amycolatopsis echigonensis JCM 21831T (AB248535)
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Amycolatopsis niigatensis JCM 21832T (AB248537)
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Amycolatopsis benzoatilytica DSM 43387T (AY957506)
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Amycolatopsis albidoflavus DSM 44639T (AJ252832)
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Amycolatopsis halotolerans NRRL B-24428T (DQ000196)
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Amycolatopsis rubida DSM44637T (AF222022)	Amycolatopsis sacchari DSM 44468T (AF223354)
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Amycolatopsis taiwanensis NBRC 102103T (DQ160215)
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94.1
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89.92

Amycolatopsis marina NBRC 104263T (EU329845)
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Amycolatopsis palatopharyngis JCM 12460T (AF479268)
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Amycolatopsis fastidiosa DSM 43855T (AB184566)
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Exhibit 4

>a1-27F
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>a1-1492R
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>a1-1492R Inv compl
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Seq A

GCTGAAGCACCTTCGGGTGTGGATGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCTGTACTTTGG
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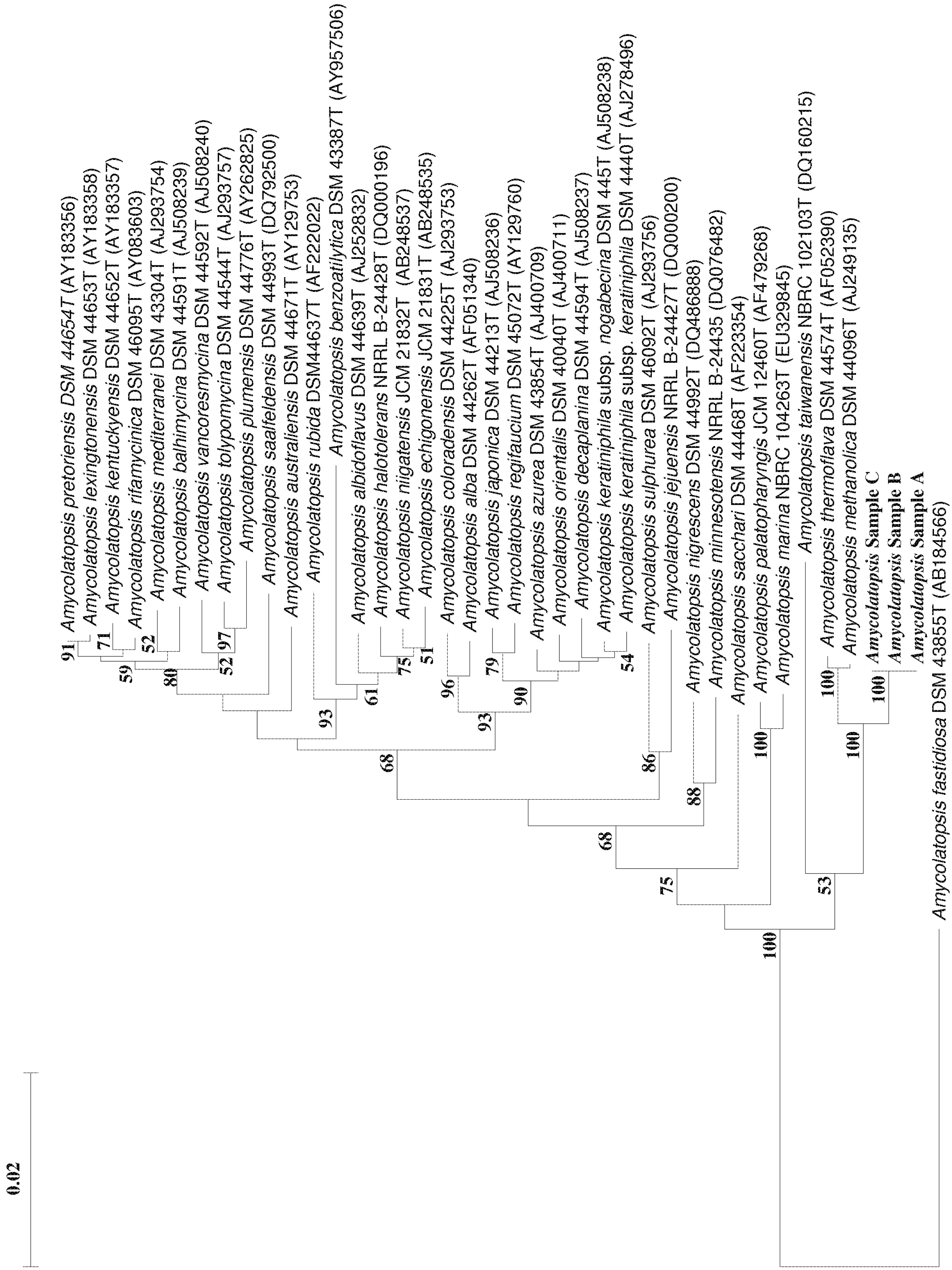
Exhibit 5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0.02

Exhibit 6

EXTERNAL MEMORANDUM

Re : Report of production trials according to US 6,133,003 to Rabenhorst et al.

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Société Anonyme au capital de 1.830.000 € N° SIRET : 389-013-491-00011 APE : 1089 Z
N° intracommunautaire : FR 20 389 013 491

1. Goal

The goal is to compare two strains :

- *Amycolatopsis* sp. DSM 9992
- *Amycolatopsis* sp. Zyl 926 (referred to also as Asp L-ii 2)

when cultivated in identical conditions, namely identical inoculating chain, fermentation process and equipment.
These two strains were evaluated for the production of vanillin by reducing to practice the process taught in US Patent 6,133,003 to Rabenhorst et al, sometimes referred to as the Symrise process in the following. At the end of the present memo, some results are given while using strain Zyl 926 (Asp Lii-2) according to the process taught in US Application N° 10/550,945 to Heald et al, referred to as the Safisis process.

2. Material and Methods

2.1 Cultures work out

The process applied in this study was according to the teaching of US Patent 6,133,003 to Rabenhorst et al "Process for the preparation of vanillin and microorganism suitable therefor".

2.1.1 Inoculating chain

- According to working Example 1 of US 6,133,003

Preparation of the preliminary culture

A 500 ml conical flask with a side baffle was filled with 100 ml of medium, comprising 1 g of malt extract, 0,4 g of glucose and 0,4 g of yeast extract and made up to 100 ml with water and then sterilized for 20 min at 121°C. After cooling, the flask was inoculated with 200 µl of a frozen glycerol culture of Amycolatopsis sp. DSM 9992 or Zyl 926 (Asp L-ii 2). The cultures were incubated on a rotary shaking machine at 45 °C and 100 rpm. After 24 h, these cultures were used to inoculate the production medium.

- Preliminary culture medium preparation

Product	Concentration
Glucose	**4 g/Kg**
Malt extract	**10 g/Kg**
Yeast extract	**4 g/Kg**
H_2O	**q.s.p**
pH not adjusted	
Sterilization 20' at 121°C	

- Preculture inoculation

*Inoculation of 100 mL with 0,2 mL from the content of a cryotube (frozen glycerol culture)

*Incubation (shaking machine) : 45°C - 100 rpm - 24h

2.1.2 Cultures in fermenters (maximum working volume = 15L)

- **Stage production according to working Example 3 of US 6,133,003**

Vanillin Production in a 10 L fermenter.

5 l of culture medium (4 g/l of glucose, 10 g/l of malt extract and 6 g/l of yeast extract) were sterilized in a fermeter and, after cooling, were inoculated with 100 ml of a seed culture of DSM 9992 or Zyl 926 (Asp L-ii 2) according to Example 1.

According to working Example 3, the culture conditions were : 37°C, 500 rpm, 5 l of air/min, 12,5 h after inoculation, 1.634 kg of an approximately 3.7% strength ferulic acid solution (60.2 g of ferulic acid) were added. After 17.5 h, a further 4.397 kg of an approximately 3.7% strength ferulic acid solution (164.72 g of ferulic acid) were pumped in over a period of 10 h.
While trying to reproduce these very same parameters, I had to adapt some of the above data to make it work and arrive at the following parameters :

- Culture parameters

	GROWTH	BIOCONVERSION	BIOCONVERSION
Inoculation	100 mL	-	-
Temperature	37°C	37°C	37°C
Medium quantity	5 Kg	1.6 Kg	4.4 Kg
Culture condition	Batch	Batch (isolated addition of ferulic acid)	**Fed-batch during 10 h (7.2 mL/min)**
Duration	12.5h	5h	14.5h
pO_2 Regulation	50% (not mentioned in the example 3 of the patent)		
Aeration	5 L/min	7 L/min	7 L/min
Stirring	400 à 750 rpm		
pH regulation	8 ± 0.5 (not mentioned in the example 3 of the patent but in the spec.)		
Surpression	No		
End of culture	-	-	32 h

- Fermenters preparation

Product	Concentration
Glucose	**4 g/Kg**
Malt extract	**10 g/Kg**
Yeast extract	**6 g/Kg**
Propylene glycol P2000	**0.35 g/Kg**
H_2O	**q.s.p**
5 Kg of medium in the fermenter *in situ* sterilization	

- Ferulic acid solution

Product	Concentration
Ferulic acid	**3.75%**
pH adjustment : 7.4 with a sodium hydroxyde solution*	

*preliminary tests to dissolve ferulic acid in water were carried out in vain. I had to add sodium hydroxide otherwise I could not get any homogeneous solution.

Detailed sequence of addition of the ferulic acid solution :

- At T12,5 h of culture, 1,6 kg of the ferulic solution were added in the fermenter

- For the semi-continuous step which starts at 17,5 h of culture, 1,3 kg of solution were prepared to feed in the fermenter. It was necessary to reload the solution every 2,5 h because a degradation of ferulic acid is observed when the solution is prepared more than 3 h in advance. The flow rate is 7,2 ml/min during 10 h, allowing to add 4,4 kg of ferulic acid solution.

- After 32 h, the fermentation was terminated.

2.2 Culture follow up

2.2.1 Culture parameters

For each culture, parameters were monitored as follows :

- pO2 (dissolved oxygen in the medium) which allows to visualize the bacterial growth by the more or less important oxygen demand. This parameter is controlled by regulating aeration and/or agitation of the medium to a certain level in order to secure optimal growth conditions
- pH is regulated to favor bacterial growth and the production of metabolites of interest
- Solutions used for pH regulation [sulfuric acid (H_2SO_4) and/or sodium hydroxyde (NaOH)]

2.2.2 Bacteriological follow up

A bacterial follow up was realized in order to be ascertain the absence of any contamination.

2.2.3 HPLC Analyses

For each culture, several samples were withdrawn at mentioned times and frozen until analyzed :

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http : www.safisis.com
Société Anonyme au capital de 1.830.000 € N° SIRET : 389-013-491-00011 APE : 1089 Z
N° intracommunautaire : FR 20 389 013 491

T0
T4
T8
T12,5 after spot addition of ferulic acid
T17,5 after the starting of the semi-continuous culture
T20 before reloading of the ferulic acid solution
T22,5 before reloading of the ferulic acid solution
T25 before reloading of the ferulic acid solution
T27,5 before termination of the semi-continuous culture
T32

Production of vanillin, vanillic acid, vanillic alcohol, guaiacol and of the ferulic acid consumption were assayed by HPLC (high pressure liquid chromatography) and UV detection, consumption of glucose was tested through HPLC and refractometry.

Two dilutions were made for each sample in order to be sure to be in the detection range of the metabolites.

3. Results

3.1 Culture follow up

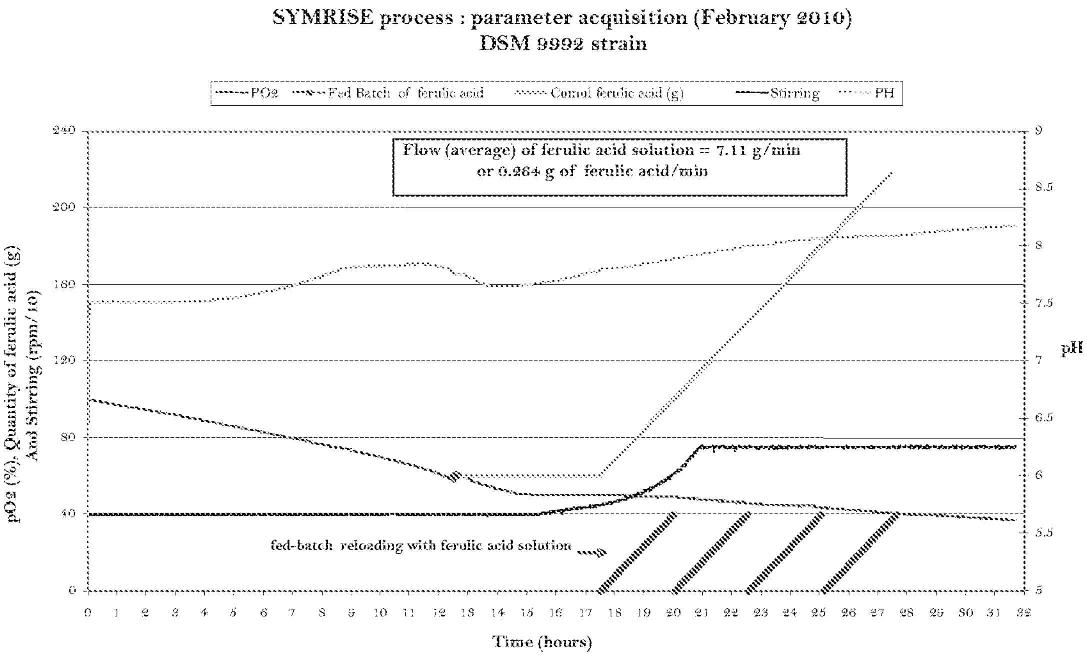
3.1.1 DSM 9992

As illustrated by Figure 1, growth phase went fine, as shown by the pO2 which decreased from 100 % to 57 % between T0 and T12,5 h. This pO2 value had slightly risen during the spot addition of ferulic acid (to 61 %) then decreased again regularly to reach 36 % at the termination of the culture. The 50 % instruction value for pO2 could not be maintained, though agitation was set at a high limit but pO2 did not drop under 30 %, thus oxygen supply has been sufficient throughout the culture duration.

Regarding pH regulation, the addition of acid and/or base solution was not necessary, let alone at the starting of the culture to restore the pH at 8,0 ± 0,5.

At T24 h, I observed a high foam production necessitating the addition of antifoam propylene glycol P2000.

At the termination of the culture, the culture medium was of a yellow/orange color, greasy –probably due to the presence of antifoam) and I noticed very high levels of clove odor/spice, showing *a priori* that bioconversion practically did not occur.



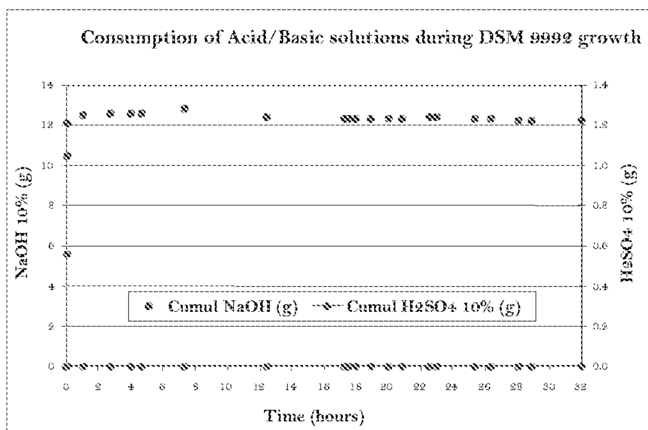
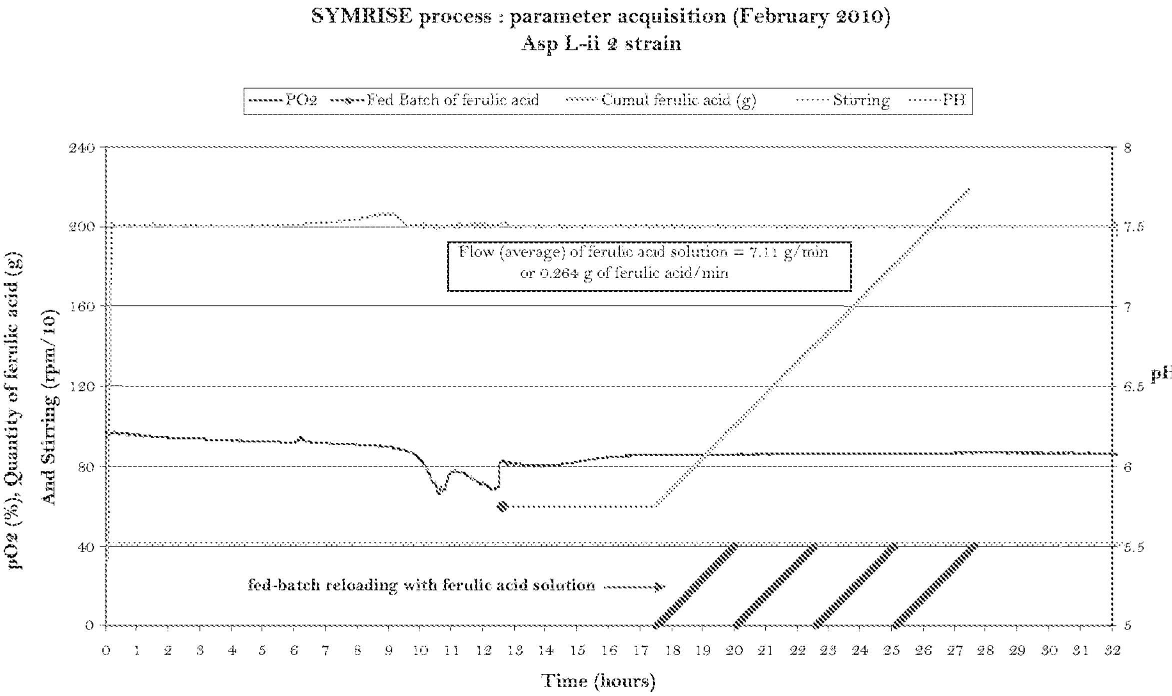


Figure 1 : Parameter acquisition for DSM 9992 strain cultivated according to Symrise process (US 6,133,033) (Cumul = Total)

3.1.2 Zyl 926 (Asp L-il 2)

As shown in Figure 2, this experiment took place with noticeable differences compared to the previous one with strain DSM 9992 :

- pO2 decreased regularly during growth phase then went over 80 % after the spot addition of ferulic acid
- pH regulation by base solution addition only after addition of ferulic acid during the bioconversion (total amount added : 120g of NaOH 10%).



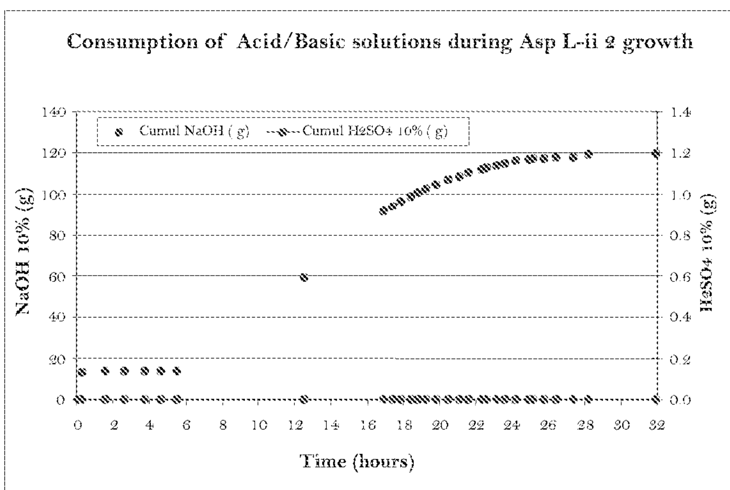


Figure 2 : Parameter acquisition for the Zyl 926 (Asp L-ii 2) strain cultivated according to the Symrise process (US 6,133,033)

3.1.3 Cultures comparison

Table 1 illustrates the principal data :

Table 1 : Growth comparison of DSM 9992 and Zyl 926 (Asp L-ii 2) strains

	pO2	pH	pH regulation with sodium hydroxide 10%	pH regulation with sulfuric acid 10%	Medium composition at the end of the culture
DSM 9992	**Growth phase** (between 0 - 12 h): decrease from 100% to 57% **Bioconversion phase** (between 12 - 32 h) : increase to 61% after ferulic acid addition, then decrease until the end (36% at 32h)	Low initial pH (5.4) → addition of 15g of sodium hydroxide 10% to adjust pH at 8.0 ± 0.5	**Bioconversion phase** (between 12 - 32 h) : NO	**NO**	Oily medium (due to antifoam), yellow/orange, strong smell like a clove
Zyl 926 (Asp L-ii 2)	**Growth phase** (between 0 - 12 h): continuous decrease from 100% to 69% **Bioconversion phase** (between 12 - 32 h) : increase to 85% after ferulic acid addition, then static until the end	No need to adjust initial pH	Continuous addition of sodium hydroxide 10% during bioconversion phase (quantity : 120g)	**NO**	Orange medium, smell like butter

3.2 Analytical results

Table 2 allows to compare the results after the termination of the culture for the two strains :

Table 2 : End product concentrations for Zyl 926 (Asp L-ii 2) and DSM 9992 strains when cultivated according to Example 3 of US 6,133,003

Strain	Vanillic alcohol (g/Kg)	Vanillic acid (g/Kg)	Vanillin (g/Kg)	Guaiacol (g/Kg)
Asp L-ii 2	0	0	0	0
DSM 9992	**<0.1**	**0.3**	**<0.1**	**0**

Chromatograms below clearly indicate that there was an accumulation of ferulic acid over time, whatever strain was used.
No metabolite of interest was detected for strain Zyl 926 (Asp L-ii 2) as illustrated in Figure 3 below, and as shown in Figure 4 below, almost none in the case of strain DSM 9992 : under 0,1 g/kg (0,1 g/l) regarding vanillin, which value should be interpreted as follows : practically no vanillin was produced contrary to the statement in Example 3 of US 6,133,003.

Moreover, at T17,5 h-T20 h-T22,5 h-T25 h and T27,5 h, analysis of intermediary samples was conducted and showed no evidence of the presence of metabolites which could have been produced and then eventually degraded during the cultivation period.

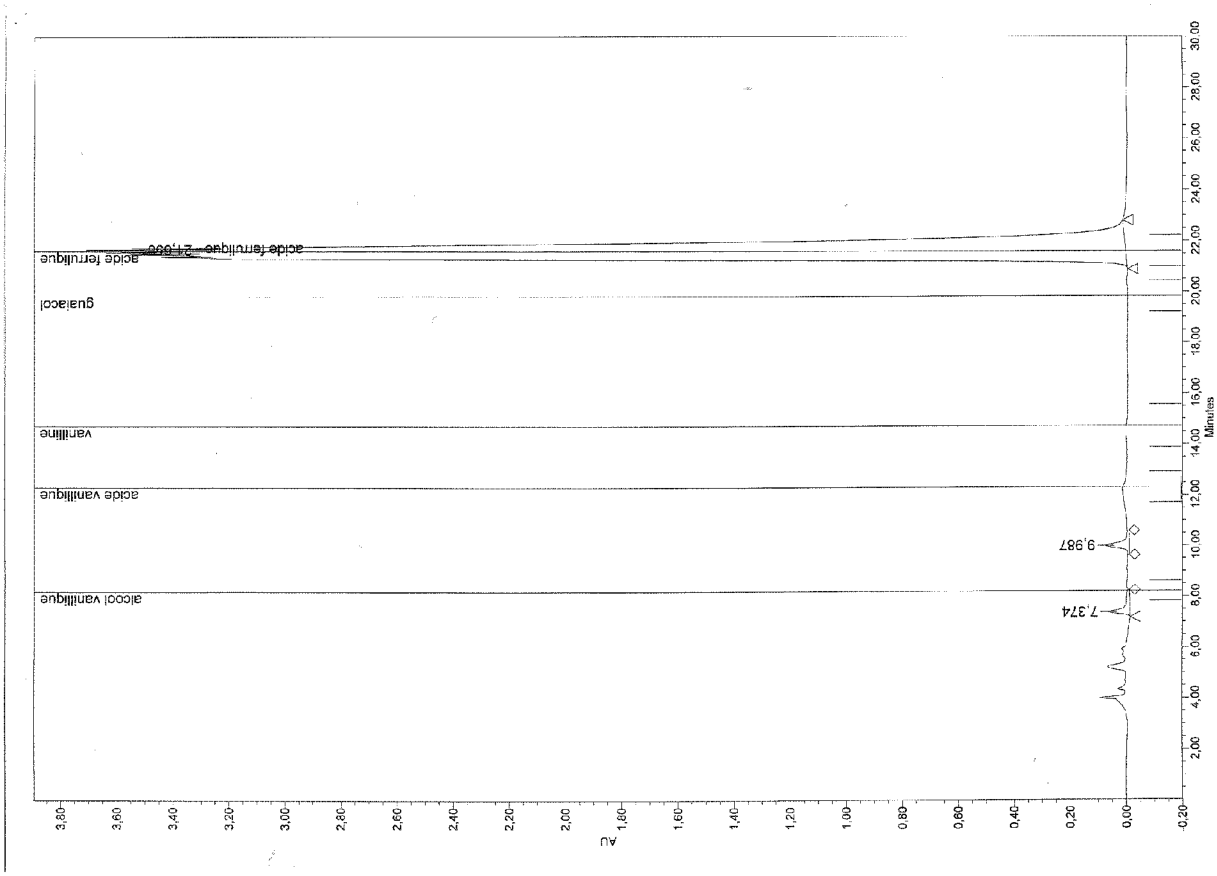


Figure 3 : Chromatogram/Symrise process (US 6,133033) with Zyl 926 strain (Asp L-ii 2) – sample corresponding to the end of the culture (T32 h)
[French/English translation: alcool vanillique/vanillic alcohol – acide vanillique/vanillic acid – vanilline/vanillin – guaiacol/guaiacol – acide férulique/ferulic acid]

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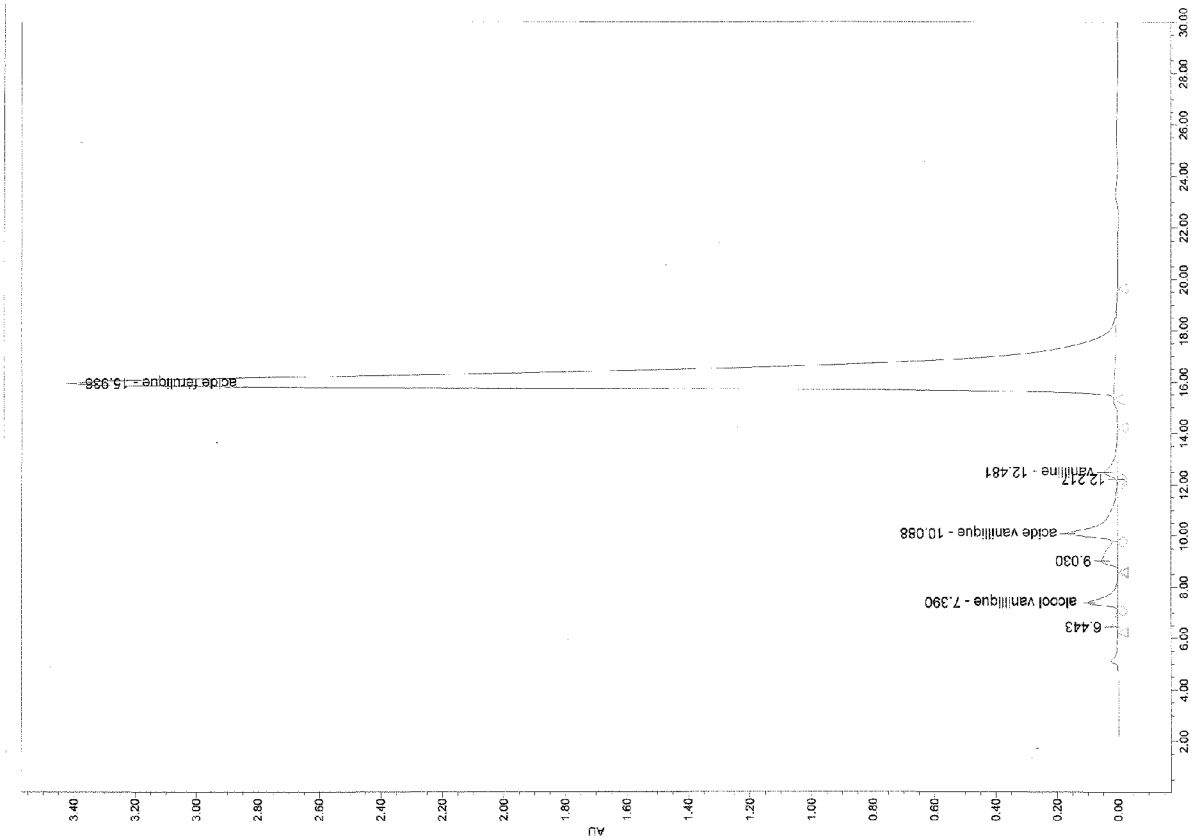

Figure 4 : Chromatogram/Symrise process (US 6,133,033) with DSM 9992 strain – sample corresponding to the end of the culture (T32 h)

[French/English translation: alcool vanillique/vanillic alcohol – acide vanillique/vanillic acid – vanilline/vanillin – guaiacol/guaiacol – acide férulique/ferulic acid]

Table 3 illustrates the accumulation profile of ferulic acid during cultures.

Table 3 : Accumulation profile of ferulic acid in the culture medium

Sample	Ferulic acid Theoretical quantity added (g)	Ferulic acid Theoretical quantity cumulated* (g)	Ferulic acid Theoretical concentration* (g/Kg)	Ferulic acid HPLC results (g/Kg)
T12.5 after addition of ferulic acid solution	60.2 g	-	9.1 g/Kg	8.9 (± 0.3)**
T20 before fed-batch reloading	48.75 g	108.9 g	13.8 g/kg	15.0 (± 2.4)**
T22.5 before fed-batch reloading	48.75 g	157.7 g	17.1 g/Kg	17.3 (± 1.8)**
T25 before fed-batch reloading	48.75 g	206.5 g	19.7 g/Kg	18.5 (± 1.6)**
T27.5 at the end of fed-batch culture	48.75 g	255.3 g	21.6 g/Kg	21.1 (± 0.8)**
T32				19.8 (± 1.1)**

*Two hypothesis :
- no addition of sodium hydroxide to regulate pH
- no consumption and/or loss of ferulic acid between fed-batch reloading
** Result average (g/Kg)

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As illustrated by theoretical calculations reported in Table 3 above, bioconversion phase was conducted as taught in US patent 6,133,003 but ferulic acid accumulated and its consumption and degradation after introduction in the culture medium are negligible.

4. Conclusion

Though conducted with identical culture conditions, the above analytical results allow to conclude that strain DSM 9992, claimed in US 6,133,003 and Zyl 926 (Asp L-ii 2), claimed in US Application N° 10/550,945, have shown a different behavior when grown in fermenters. Besides, in both cases growth phase went well.
Moreover, HPLC analysis were performed in order to quantify metabolites of interest, namely vanillin, guaiacol, vanillic alcohol and vanillic acid expected to be produced in such conditions.
These trials results allow us to conclude that no ferulic acid reacted and the latter accumulated in the culture medium during the course of the culture.

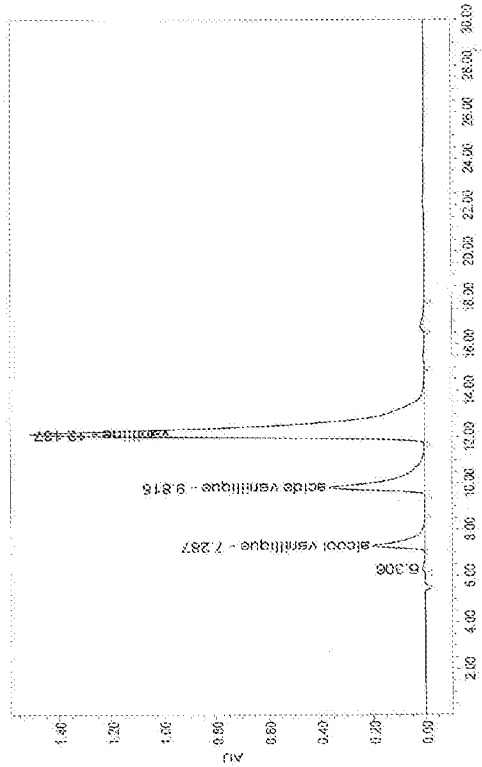
These were unexpected results since the parameters applied in the present study were those taught in working Examples of the document US 6,133,003 to Rabenhorst et al., "Process for the preparation of vanillin and microorganisms suitable therefor".

Moreover we, present engineers who signed the appended affidavits, made all reasonable attempts to use the complete teaching of this specification and not only the Example wording. See for example to name but a few "adjustments", the preparation of ferulic acid and also pH and pO_2 regulation and antifoam necessary usage.

As a conclusion, and based on the forgoing facts, this prior art, namely US 6,133,003 is of a non operative kind, the reason being that a person skill in the art by repeating/reproducing its teaching not only does not get the expected results but rather gets essentially no production of the desired product, namely vanillin. To arrive at this production, one should "invent" another process.

Accordingly, US 6,133,003 is not at all pertinent, and by far, the production of vanillin as taught and claimed by the inventors of US Application N° 10/550,945 is not obviously derivable therefrom.

By way of comparison below is shown in Figure 5, a chromatogram obtained by working out the process taught in US Application N° 10/550,945 to Heald et al, namely the "Safisis process", using Zyl 926 (Asp L-ii 2) : ferulic acid was spent and the vanillin peak is predominant (14 g/l).



	Peak Name	RT	Area	% Area	Height	Amount	Units	Dilution	Vial
1		6.306	202786	0.32	11303			20.01390	45
2	alcool vanillique	7.287	4168799	6.53	196807		g/kg	20.01390	45
3	acide vanillique	9.815	10536140	16.49	362149	1.439	g/kg	20.01390	45
4	vanilline	12.137	48571010	76.03	1508580	14.028	g/kg	20.01390	45
5	acide ferulique	15.377						20.01390	45

Figure 5 : Chromatogram/Safisis process (US App. N° 10/550,945) with Zyl 926 strain (Asp L-ii 2) - sample withdrawn at the end of the culture

[French/English translation: alcool vanillique/vanillic alcohol – acide vanillique/vanillic acid – vanilline/vanillin – guaiacol/guaiacol – acide féruliqueiferulic acid]

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